pH-jump-induced phosphorylation of ADP in the cyanobacterium *Anacystis nidulans*

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The low ATP levels in dark anaerobic cells of the cyanobacterium *Anacystis nidulans* more than doubled within 5 s after rapid addition of HCl shifting external pH from 9.0 to 4.5. Steady-state levels of ATP and intracellular pH remained constant at 0.95 ± 0.15 nmol/mg dry weight and 6.9 ± 0.3, respectively. ΔpH-induced ATP synthesis was inhibited by dicyclohexylcarbodiimide and carbonyl cyanide *m*-chlorophenylhydrazone but not by carbon monoxide. According to our results the cytoplasmic membrane of *A. nidulans* has to be regarded as an energy-transducing membrane bioenergetically similar to the thylakoid membrane.

pH gradient *Protonmotive force* *Reversible H^+-ATPase* *ATP synthesis* *Cytoplasmic membrane* Anacystis nidulans

1. INTRODUCTION

The chemiosmotic theory predicts that a proton electrochemical gradient ($\Delta\mu_{H^+}$) or protonmotive force (p.m.f.) across energy-transducing membranes ultimately powers ATP synthesis by a reversible ATPase present in the membrane [1]. Besides a membrane potential ($\Delta\psi$) the pH concentration gradient (ΔpH) is the second essential component of the protonmotive force. Jagendorf and Uribe [2] were the first to demonstrate that an artificial pH gradient (acidic inside) imposed across thylakoid membranes of isolated chloroplasts can drive ATP synthesis. Since then, artificially imposed pH gradients have been shown to drive ATP synthesis also in mitochondria [3], submitochondrial vesicles [4], bacterial vesicles [5], intact bacteria [6–8] and artificial lipid vesicles reconstituted with a reversible ATPase [9]. The data accumulated lend much support to the chemiosmotic theory [10]. Yet, in vivo, localized proton gradients more closely associated with the energy-transducing membrane may be involved rather than bulk phase gradients which, of course, do function in vitro [11].

In cyanobacteria little is known about energy-transducing properties of the cytoplasmic membrane [12–14]; it is still impossible to obtain reliable preparations of pure cytoplasmic membrane separated from thylakoid membranes which, in turn, certainly are responsible for most of the photosynthetic electron transport activities [12–14]. However, the data obtained with crude particle preparations (containing both cytoplasmic and thylakoid membranes) or intact cells of *Anacystis nidulans* have suggested that the cytoplasmic membrane, in addition to the thylakoid membrane, is a site of respiratory electron transport resulting in primary proton extrusion [14–16]. The proton gradient thus established

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; Hepes, *N*-(2-hydroxyethylpiperazine)-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); Taps, tris(hydroxymethyl)methylaminopropanesulfonic acid; DMO, 5,5-dimethyloxazolidine-2,4-dione; TPP^+, tetraphenylphosphonium ion

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across the cytoplasmic membrane in vivo (alkaline inside) could power ATP synthesis through a reversible ATPase present in the membrane. In fact, ATP synthesis was observed when an artificial ΔpH (alkaline inside) was imposed across the cytoplasmic membrane of energy-depleted cells of *A. nidulans* as shown in this communication.

2. MATERIALS AND METHODS

Axenic cultures of *A. nidulans* (strain 1402/1, Göttingen) were grown to the late logarithmic phase at 38°C, 1 kW/m^2 warm white fluorescent light and a constant pH of 8.2, and harvested as in [17]. Cells were washed twice with 5 mM Tris–Hepes buffer (pH 9.0) and finally resuspended therein at a cell concentration of 50 μl packed cells (about 15 mg dry weight/ml; acid pulse experiments). For the determination of steady-state levels of intracellular ATP and pH the cells were washed and resuspended in 25 mM citrate, Mes, Pipes, Hepes or Taps buffer adjusted to the appropriate pH with KOH. ATP was determined with a luciferin-luciferase assay as in [18,19]. Intracellular pH was measured by use of flow dialysis [20] following the distribution between intracellular and extracellular space of ^{14}C-labelled aspirin, DMO and methylamine, respectively, depending on the extracellular pH; similarly $\Delta\psi$ was calculated from the distribution of ^{3}H-labelled TPP$^+$ [21]. Details of these determinations in *A. nidulans* will be published elsewhere (Schmetterer, Nitschmann and Peschek, unpublished). Intracellular volume of the cells was taken as 70% of the packed cell volume [22]. Acid pulses were applied in the form of a few microliters of anaerobic 0.1 N HCl rapidly injected into 20 ml cell suspension (initial pH 9.0); light and oxygen were carefully excluded throughout the experiments. Oxygen and pH in the suspension were continuously monitored with a YSI oxygen electrode (model 53) and a pH electrode (Seibold, Vienna), respectively, both connected to a Rikadenki Multi-pen Recorder. The temperature was 35°C in all experiments. The values given in the figures and the table are the means of at least 5 independent determinations, standard deviations among individual data ranging from 10–15% of the corresponding mean.

3. RESULTS AND DISCUSSION

Fig.1 shows the net increase in intracellular ATP measured 5 s after shifting dark anaerobic suspensions of *A. nidulans* from pH 9.0 to the final pH values indicated on the abscissa (curve a). At the same time it is seen that the steady-state ATP concentration in dark anaerobic cells (measured 20 min after the onset of dark anaerobic incubation) remained virtually unaffected by external pH (curve b). Intracellular pH likewise remained nearly constant (at 6.9 ± 0.3) over a range of external pH values between 4.3 and 9.2 (not shown; Schmetterer, Nitschmann and Peschek, unpublished). Therefore the increase in ATP content of dark anaerobic cells subjected to acid pulses does not reflect possibly different steady-state ATP levels or changes of ΔpH across the intracellular (thylakoid) membranes which do function in photosynthetic energy conservation. Rather, the elevated ATP levels (fig.1a) are caused by net synthesis of ATP through a cytoplasmic membrane-bound reversible ATPase utilizing the proton concentration gra-

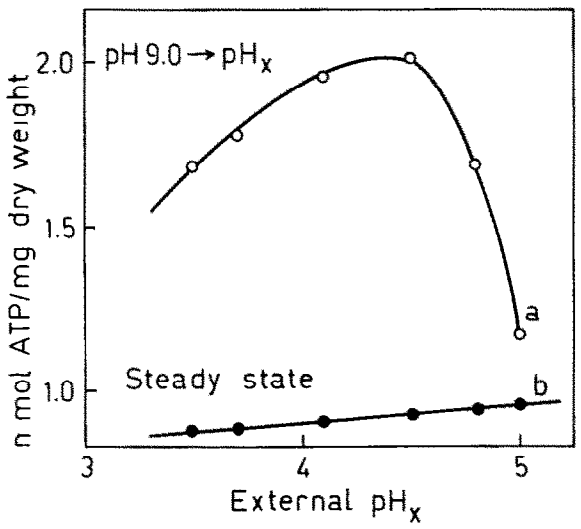

Fig.1. (a) Elevated ATP levels in dark anaerobic *A. nidulans* measured 5 s after rapid application of acid pulses shifting external pH from 9.0 to pH$_x$. (b) Steady-state concentrations of ATP in dark anaerobic *A. nidulans* measured after 20 min of preincubation. Internal pH was 6.9 + 0.3 independent of external pH. Anaerobiosis was established, and maintained, by sparging the suspensions with oxygen-free nitrogen or with carbon monoxide which gave the same results.

dient artificially imposed. The same conclusion is derived from the transient changes of intracellular adenylate pool sizes (fig.2a,b) which do not conform to simple adenylate kinase action. Mechanistically, any proton translocation through the cytoplasmic membrane-bound ATPase appears to be tightly coupled to ATP synthesis or hydrolysis by the enzyme thus explaining the sudden, and likewise transient, drop of intracellular ATP after an acid/base shift (fig.2b). During the latter transition protons may be expelled from the cell partly through the ATPase thereby provoking

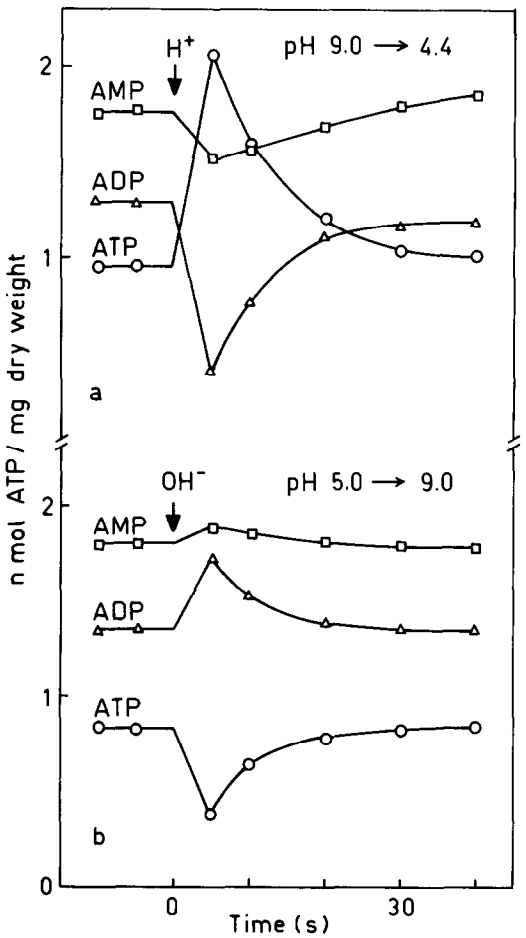

Fig.2. Kinetics of the change of adenylate levels in dark anaerobic cells of *A. nidulans* shifted from external pH 9.0 (5 mM Tris–Hepes buffer) to 4.4 by rapid addition of 0.1 N HCl (a) and from external pH 5.0 (5 mM Mes–Hepes buffer) to 9.0 by rapid addition of 0.5 M Tris base (b) so as to reach the final pH values indicated. ATP, ADP and AMP were determined as in [18,19].

ATP hydrolysis even without any thermodynamic need. At the same time this demonstrates the fundamental reversibility of the cytoplasmic membrane-bound enzyme capable of functioning as a proton-translocating ATPase under physiological conditions [19]. That the pH-jump-induced changes of intracellular adenylate levels were highly transient, steady-state levels being re-established within at most 60 s after the pulse (fig.2), is attributed to the energetic homeostasis characteristic of living cells.

The effect of inhibitors (table 1) was consistent with the assumption that the ΔpH-induced increase of intracellular ATP-concentration in dark anaerobic cells of *A. nidulans* as shown in fig.1a,2a is due to a reversible ATPase utilizing proton concentration gradients across the cytoplasmic membrane (alkaline inside) for ATP synthesis: DCCD, which is known to plug the proton channel of membrane-bound ATPase, completely abolished any ΔpH-dependent ATP synthesis, and the same was observed in the presence of the uncoupler CCCP which short-circuits the proton concentration gradient across the membrane (table 1). The latter result makes it unlikely that, in *A. nidulans*, mere protonation of the ATPase may elicit ATP synthesis through conformational changes of the enzyme as was recently proposed for mitochondria [23]. The involvement of functional sulfhydryl groups of the enzyme is in-

Table 1

Influence of inhibitors on pH-jump-induced increases of intracellular ATP levels in dark anaerobic cells of *A. nidulans*

Inhibitor	pH 9.0⟶4.5	pH 9.0⟶4.8
None	225	181
Carbon monoxide	225	180
5 μM CCCP	146	118
50 μM CCCP	100	100
50 μM DCCD	135	109
100 μM DCCD	100	100
30 μM *p*-chloro-mercuribenzoate	125	110

Inhibitors were added 5–10 min before application of the acid pulse (shifting external pH from 9.0 to 4.5 and 4.8, respectively). ATP levels reached within 5 s after the jump are given as % of corresponding steady-state levels

dicated by the effect of *p*-chloromercuribenzoate (table 1) which conforms to previous findings with membrane-bound ATPases [24]. Complete lack of inhibition by carbon monoxide, which is a potent inhibitor of cyanobacterial terminal oxidases [25,26], safely excludes any ATP formation by oxidative phosphorylation [18,19] due to trace amounts of oxygen inadvertently introduced in the course of acid pulse experiments.

4. CONCLUDING REMARKS

Fig.3 attempts to summarize our present knowledge of membrane-bound bioenergetic mechanisms in cyanobacteria: Thylakoid membranes are shown to contain an electron transport system capable of serving both respiration and photosynthesis through common carriers, viz. plastoquinone and the cytochrome f/b_6 complex [27,28]. In addition, the cytoplasmic membrane (of *A. nidulans*) appears to be endowed with respiratory functions [14–19,29]. In particular, a cytoplasmic membrane-bound cytochrome oxidase, presumably of aa_3-type [25,26,30], might establish a transmembrane proton electrochemical gradient through active proton extrusion [15,16]. The resulting ΔpH, in turn, can be used by a similarly cytoplasmic membrane-bound ATPase to drive ATP synthesis as discussed here. Estimation of the total protonmotive force resulting across the cytoplasmic membrane upon a shift from pH 9.0 to 4.4 shows that ATP formation indeed should be possible under these conditions since p.m.f. = $\Delta\psi - 61$. ΔpH = -270 mV at 35°C using $\Delta\psi = -100$ mV, $pH_{in} = 7.2$, and assuming $\Delta\psi$ and ΔpH to remain unchanged during the first 5 s of the experiment (Schmetterer, Nitschmann and Peschek, unpublished). Yet, another part of the ΔpH may directly power proton/cation antiporters in the cytoplasmic membrane (fig.3). Such function of an H^+/Na^+-antiporting system was recently inferred from lowered net efficiencies of oxidative phosphorylation displayed by sodium-containing cells of *A. nidulans* [18,19].

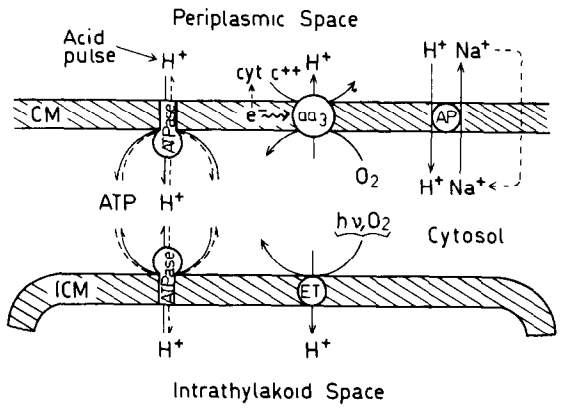

Fig.3. Scheme of membrane-bound bioenergetic functions in *A. nidulans*. CM, cytoplasmic membrane; ICM, intracytoplasmic (thylakoid) membrane; ET, electron transport; aa_3, terminal oxidase (also present in thylakoid membranes; not shown); AP, antiporting system; $e^- \longrightarrow$, electron donation to the cytoplasmic membrane-bound terminal oxidase through hitherto unknown intermediates, among them possibly a periplasmic *c*-type cytochrome [31].

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REFERENCES

[1] Mitchell, P. (1968) Chemiosmotic Coupling and Energy Transduction, Glynn Research, Bodmin.
[2] Jagendorf, A.T. and Uribe, E. (1966) Proc. Natl. Acad. Sci. USA 55, 170–177.
[3] Rossi, E. and Azzone, G. (1970) Eur. J. Biochem. 12, 319–327.
[4] Thayer, W.S. and Hinkle, P.C. (1975) J. Biol. Chem. 250, 5330–5335.
[5] Apel, W.A., Dugan, P.R. and Tuttle, J.H. (1980) J. Bacteriol. 142, 295–301.
[6] Maloney, P.C., Kashket, E.R. and Wilson, T.H. (1974) Proc. Natl. Acad. Sci. USA 71, 3896–3900.
[7] Wilson, D.M., Alderete, J.F., Maloney, P.C. and Wilson, T.H. (1976) J. Bacteriol. 126, 327–337.
[8] Maloney, P.C. and Wilson, T.H. (1975) J. Membrane Biol. 25, 285–310.
[9] Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1977) J. Biol. Chem. 252, 2956–2960.
[10] Nicholls, D.G. (1982) Bioenergetics – An Introduction to the Chemiosmotic Theory, Academic Press, London.

[11] Williams, R.J.B. (1978) Biochim. Biophys. Acta 505, 1–44.
[12] Stanier, Y. and Cohen Bazire, G. (1977) Annu. Rev. Microbiol. 31, 225–274.
[13] Carr, N.G. and Whitton, B.A. (1982) The Biology of the Cyanobacteria, 2nd edn, Blackwell, Oxford.
[14] Peschek, G.A. (1983) in: Subcellular Biochemistry (Roodyn, D.B. ed) vol.10, Plenum Press, New York, in press.
[15] Peschek, G.A. (1983) J. Bacteriol. 153, 539–542.
[16] Peschek, G.A., Schmetterer, G., Lauritsch, G., Muchl, R., Kienzl, P.F. and Nitschmann, W.H. (1983) in: Photosynthetic Prokaryotes: Cell Differentiation and Function (Papageorgiou, G.C. and Packer, L. eds) pp.147–162, Elsevier, Amsterdam, New York.
[17] Peschek, G.A., Muchl, R., Kienzl, P.F. and Schmetterer, G. (1982) Biochim. Biophys. Acta 679, 35–43.
[18] Nitschmann, W.H. and Peschek, G.A. (1982) FEBS Lett. 139, 77–80.
[19] Nitschmann, W.H., Schmetterer, G., Muchl, R. and Peschek, G.A. (1982) Biochim. Biophys. Acta 682, 293–296.
[20] Ramos, S., Schuldiner, S. and Kaback, H.R. (1979) Methods Enzymol. 55, 680–695.
[21] Rottenberg, H. (1979) Methods Enzymol. 55, 547–569.
[22] Dewar, M.A. and Barber, J. (1973) Planta 113, 143–155.
[23] Malenkova, I.V., Kuprin, S.P., Davydov, R.M. and Blumenfeld, L.A. (1982) Biochim. Biophys. Acta 682, 179–183.
[24] Delhez, J., Dufour, J.-P., Thines, D. and Goffeau, A. (1977) Eur. J. Biochem. 79, 319–328.
[25] Kienzl, P.F. and Peschek, G.A. (1982) Plant Physiol. 69, 580–584.
[26] Peschek, G.A., Schmetterer, G., Lauritsch, G., Nitschmann, W.H., Kienzl, P.F. and Muchl, R. (1982) Arch. Microbiol. 131, 261–265.
[27] Peschek, G.A. and Schmetterer, G. (1982) Biochem. Biophys. Res. Commun. 108, 1188–1195.
[28] Peschek, G.A. (1983) Biochem. J. 210, 269–272.
[29] Peschek, G.A., Schmetterer, G. and Sleytr, U.B. (1981) FEMS Microbiol. Lett. 11, 121–124.
[30] Matthijs, H.C.P. (1983) in: Abstracts of 6th International Congress on Photosynthesis (Sybesma, C. ed) vol.1, p.219, Vrije Universiteit, Brussels.
[31] Peschek, G.A., Schmetterer, G. and Wagesreiter, H. (1982) Arch. Microbiol. 133, 222–224.